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14. ABSTRACT
Bacteria that can both degrade and sense pollutants using chemotaxis may be more efficient in biodegradation. Ralstonia sp. U2 mineralizes naphthalene, and Acidovorax sp. JS42 utilizes the man-made compound 2-nitrotoluene (2NT). Capillary assays were performed with wild-type and catabolic and regulatory mutants of these strains. The results showed that wild-type Ralstonia sp. U2 is chemotactic to naphthalene, the chemotactic response is induced when cells are grown with naphthalene, and insertional inactivation of nagR, which encodes the transcriptional activator of the naphthalene catabolic genes, had no affect on chemotaxis to naphthalene. The results indicate that NagR is not required for naphthalene

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Report Title

Bacterial Chemotaxis to Naphthalene and Nitroarene Compounds

ABSTRACT

Bacteria that can both degrade and sense pollutants using chemotaxis may be more efficient in biodegradation. Ralstonia sp. U2 mineralizes naphthalene, and Acidovorax sp. JS42 utilizes the man-made compound 2-nitrotoluene (2NT). Capillary assays were performed with wild-type and catabolic and regulatory mutants of these strains. The results showed that wild-type Ralstonia sp. U2 is chemotactic to naphthalene, the chemotactic response is induced when cells are grown with naphthalene, and insertional inactivation of nagR, which encodes the transcriptional activator of the naphthalene catabolic genes, had no affect on chemotaxis to naphthalene. The results indicate that NagR is not required for naphthalene chemotaxis, and naphthalene is detected directly. 2NT-induced wild-type JS42 showed a stronger response to 2NT than uninduced cells. Mutant strains unable to degrade 2NT did not show an inducible response to 2NT, but did respond like uninduced wild type. The inducible response was lost when JS42 was exposed to a competing energy source. Thus, JS42 uses two types of 2NT taxis: 2NT-specific chemotaxis and metabolism-dependent energy taxis, in which the energy generated from 2NT metabolism is detected. The chemotactic response is constitutive, while energy taxis is induced during exposure to 2NT and other inducers of the 2NT degradation genes.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

R.E. Parales, K.-S. Ju, J. Rollefson, and J.L. Ditty. 2008. Bioavailability, transport and chemotaxis of organic pollutants. pp. 145-187. In: Microbial Bioremediation. E. Diaz, (ed.) Caister Academic Press, Norfolk, UK. (Invited Book Chapter)

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C.A Rabinovitch and R.E. Parales. Characterization of the taxis system of Acidovorax sp. strain JS42 towards 2-nitrotoluene. Annual Meeting of the West Coast Bacterial Physiologists, Pacific Grove, CA, December 2007.

C.A Rabinovitch and R.E. Parales. Characterization of the taxis system of Acidovorax sp. strain JS42 towards 2-nitrotoluene. Abstr. 108th General Meeting of the American Society for Microbiology, Boston, MA, June 2008.

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Number of Inventions:

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NAME	PERCENT_SUPPORTED
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Christine Rabinovitch	0.50
FTE Equivalent:	**0.75**
Total Number:	**2**

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NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Rebecca E. Parales	0.10	No
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

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Nancy Ngo	0.20
Amy Chi	0.20
Tiffany Louie	0.20
Danial Samar	0.10
FTE Equivalent:	**0.90**
Total Number:	**5**

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The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	5.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	2.00
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The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
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Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

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Final Report

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Bacterial Chemotaxis to Naphthalene and Nitroarene Compounds

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July 31, 2008

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Statement of the Problem Studied

The major objective of this research project has been to characterize the chemotactic responses used by bacteria to detect and respond to the presence of aromatic hydrocarbons and nitroarene compounds. These classes of chemicals are considered to be serious environmental pollutants because of their known toxic effects on humans, animals, and microorganisms. Bacteria capable of completely degrading these compounds have been identified, and have the potential for use in bioremediation processes to clean up contaminated sites. Bacterial strains that are capable of both biodegradation of specific environmental pollutants and chemotactic responses to these same chemicals may be particularly efficient in biodegradation because of their ability to locate, follow, and degrade pollutants. Chemotaxis, the directed movement of motile bacteria towards or away from specific chemicals in the environment, can increase an organism's chances of locating useful sources of carbon and energy. Chemotaxis therefore contributes to the survival and competitiveness of bacteria in the environment, and may play an important role in the biodegradation process.

A molecular analysis of the nitrobenzene and 2-nitrotoluene (2NT) degradation pathways in *Comamonas* sp. JS765 and *Acidovorax* sp. JS42 revealed that the nitroarene degradation genes appeared to have evolved from a naphthalene degradation pathway like that in *Ralstonia* sp. U2 (19, 24, 26). During this project, a putative chemoreceptor gene was identified near genes for nitrobenzene oxidation in *Comamonas* sp. JS765 (20). We subsequently identified similar genes in related gene clusters involved in the degradation of naphthalene and 2NT in *Ralstonia* sp. U2 and *Acidovorax* sp. JS42, respectively. The presence of these genes suggested that these strains might be chemotactic to naphthalene or nitroarene compounds. Preliminary results demonstrated a strong chemotactic response to naphthalene by *Ralstonia* sp. U2. At the time we began this project, no further details were known about the chemotactic response to naphthalene in *Ralstonia* sp. U2, and nothing was known about the role of the homologous chemoreceptor genes in *Comamonas* sp. JS765 or *Acidovorax* sp. JS42. In this project we characterized the chemotactic responses to naphthalene by *Ralstonia* sp. U2 and to nitroarene compounds by *Acidovorax* sp. JS42.

Materials and Methods

Bacterial strains, media, and growth conditions. *Ralstonia* (formerly *Pseudomonas*) sp. U2, which grows on naphthalene as a sole carbon and energy source, was isolated from soil from an oil refinery in Venezuela (9, 10). *Acidovorax* sp. JS42 was isolated for its ability to grow on 2NT as sole carbon, nitrogen, and energy source from soil from a nitrobenzene production facility in Mississippi (14). *E. coli* DH5α (32) was used for cloning and plasmid propagation. *E. coli* S17-1 (35) and HB101(pRK2013) (8) were used in conjugations to introduce plasmids into *Ralstonia* and *Acidovorax* strains. *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth or LB agar (32) unless otherwise indicated. Matings between *E. coli* and *Ralstonia* were carried out at 30^{o}C on LB plates for 18-24 h. Matings between *E. coli* and *Acidovorax* strains were carried out at 30^{o}C on LB plates that were made without added NaCl (LB no salt) for 24-48 h. *Acidovorax* strains were grown in minimal salts medium (MSB; (36)) containing succinate (10 mM) and/or 2NT in the vapor phase, and Balch's vitamin solution (11) without thiamine. *Ralstonia* strains were grown in MSB with 10 mM succinate, 5 mM salicylate, or naphthalene crystals. Noble agar (1.8%; Difco) was used to solidify MSB medium for plates. For plasmid selection and maintenance, antibiotics were added to the growth medium at the following concentrations unless otherwise noted: for *E. coli*, ampicillin 150 µg/ml, kanamycin 100 µg/ml, streptomycin 50 µg/ml, tetracycline 15 µg/ml; for JS42, kanamycin 50 µg/ml,

streptomycin 200 µg/ml, tetracycline 12.5 µg/ml; for U2, kanamycin 100 µg/ml, tetracycline 20 µg/ml.

Cloning of the complete* nagY *gene from* Ralstonia *sp. U2. The primers used to amplify both *nagY* from *Ralstonia* sp. U2 and *ntdY* from *Acidovorax* sp. JS42 were mcpfor 5'-CTGGATCCGGAACATCTTGCGGCTGTCATGCAG-3' and mcprev 5'-CGGGATCCGGGCAACATGTGGCTACGTCA-3' (BamHI sites underlined). Genomic DNA from *Ralstonia* sp. U2 was purified using the PUREGENE DNA purification kit (Gentra Systems, Inc. Minneapolis, MN). The PCR reaction for amplification of *nagY* contained (in 50 µl total volume) approximately 200 ng of U2 genomic DNA as template, 200 µM dNTP mixture, primers at 300 µM final concentration, and buffer 3 from the Roche Extend High Fidelity PCR kit (Roche Diagnostics Corp. Indianapolis, IN) and 2 units of the Biolase DNA polymerase (Bioline USA, Inc, Taunton, MA). The reaction was carried out with an initial denaturation at 95^{o}C for 3 min, followed by 30 cycles of 95^{o}C for 1 min, 60^{o}C for 1 min, and 65^{o}C for 2 min. A final extension was carried out at 65^{o}C for 5 min. A product of the expected size, 1.5-kb was obtained. The product was purified using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA), and ligated to XcmI-digested pTAV (5) to form pTAV-Chemo9. Both strands of the *nagY* gene were completely sequenced.

Cloning of the complete* ntdY *gene from* Acidovorax *sp. JS42. Genomic DNA from *Acidovorax* sp. JS42 was purified using the PUREGENE DNA purification kit. The PCR reaction for amplification of *ntdY* contained (in 50 µl total volume) approximately 200 ng of JS42 genomic DNA as template, 200 µM dNTP mixture, primers at 300 µM final concentration, and Buffer 2 and 2.6 units of the polymerase provided in the Roche Extend High Fidelity PCR kit. The reaction was carried out with an initial denaturation at 95^{o}C for 3 min, followed by 25 cycles of 95^{o}C for 1 min, 65^{o}C for 1 min, and 72^{o}C for 2 min. A final extension was carried out at 72^{o}C for 5 min. A product of the expected size, 1.5-kb was obtained. The product was purified using the QIAquick Gel Extraction Kit, and ligated to XcmI-digested pTAV (5) to form pTAV-MCP1. The *ntdY* gene was completely sequenced and was found to be 96% identical to *nagY*.

Construction of the* Ralstonia *sp. U2* nagY *mutant. pTAV-MCP-1 was digested with MscI, which cuts once in the middle of the cloned *ntdY* gene. pACYC184:Km was digested with HincII to obtain the 1.2-kb kanamycin resistance gene, which was purified from the gel and ligated to MscI-digested pTAV-MCP-1. The resulting plasmid was digested with BamHI and EcoRI and the inactivated *ntdY* gene was inserted into pRK415 (18) that had been digested with the same restriction enzymes. This plasmid (pRK415-MCP::Km) was introduced into *Ralstonia* sp. U2 by triparental mating on LB plates at 30^{o}C by mixing DH5α(pRK415-MCP::Km), HB101(pRK2013) (8) and *Ralstonia* sp. U2. Mating mixes were plated on MSB containing 10 mM succinate and 100 µg/ml kanamycin, and the resulting kanamycin-resistant U2 strains were screened for loss of the plasmid based on tetracycline sensitivity, which indicates that a double crossover had occurred, replacing the wild-type gene with the inactivated gene. The kanamycin insertion in *nagY* in the mutant strain (U2*nagY*::km) was confirmed by PCR amplification of the *nagY* gene with the primers mcpfor and mcprev and genomic DNA prepared from the mutant strain. The resulting PCR product was approximately 2.5-kb in contrast to the 1.5-kb product from the wild type. The mutation was further confirmed by Southern blotting (data not shown). We attempted to use the pRK415-MCP::km construct to introduce a kanamycin insertion into the *ntdY* gene in *Acidovorax* sp. JS42, but although the plasmid was transferred to JS42, we were never able to obtain the double crossover despite numerous attempts.

Construction of the* Ralstonia *sp. U2* nagR *mutant. The same construct that was used to inactivate *ntdR* in *Acidovorax* sp. JS42 (pDTG957, which is pRK415 carrying *dntR*::Km

(20)) was used to generate a *nagR* mutation in *Ralstonia* sp. U2 since the *dntR*, *ntdR* and *nagR* genes are so close in sequence (98% identity). Matings between S17-1 (35) carrying pDTG957 and *Ralstonia* sp. U2 were carried out on LB plates at 30^{o}C. The mating mix was plated on MSB containing 10 mM succinate and 100 µg/ml kanamycin, and the resulting kanamycin-resistant U2 colonies were screened for loss of the plasmid based on tetracycline sensitivity. The kanamycin insertion in *nagR* in the mutant strain was confirmed by PCR amplification of the *nagR* gene with the primers ntdR-F1 (5'-GCGGGAAGCTTATGGATCTGCGCGACATCGACTTG-3') and ntdR-R1 (5'-GGCGTCTAGATTATGCTTCAGAGAAAAGCTCGACG-3') with genomic DNA prepared from the mutant strain (U2*nagR*::Km). The resulting PCR product was approximately 2.4-kb in contrast to the 0.9-kb product from the wild type.

Construction of the* Acidovorax *sp. JS42* ntdAc *mutant. pDTG850 (pUC18 carrying *ntdAaAbAcAd* from *Acidovorax* sp. JS42 (28)) was digested with KpnI, which cuts once in the *ntdAc* gene, and blunt ends were generated with T4 polymerase (21). pACYC184::Km was digested with HincII, and a 1.3 kb fragment carrying the kanamycin resistance gene was purified from the gel using the Qiagen kit, and ligated into linearized pDTG850. An ampicillin and kanamycin resistant clone with the appropriate insert was verified by digestion with PstI and BamHI. The SacI-EcoRI fragment from pDTG850-Km (carrying *ntdAaAbAc::*Km*Ad*) was moved into SacI-EcoRI-digested pRK415. Ampicillin-sensitive, kanamycin- and tetracycline-resistant strains were screened for plasmids carrying the correct SacI-EcoRI insert. The plasmid, pRK415-850-Km was used to transform *E. coli* S17-1 (35). S17-1(pRK415-850-Km) was mated with JS42 on LB no salt plates overnight at 30^{o}C. Mating mixes were resuspended in MSB medium, washed once and plated onto selective medium: MSB containing 10 mM succinate, 50 µg/ml kanamycin and vitamins. Kanamycin-resistant strains were grown in liquid medium of the same composition through several transfers, and plated on plates of the same type. Kanamycin-resistant, tetracycline-sensitive colonies were identified and tested for the ability to grow with 2NT. The resulting strain, JS42*ntdAc::*Km, was unable to grow on 2NT, but retained the ability to grow with 3-methylcatechol. Genomic DNA was purified from JS42*ntdAc::*Km using the PUREGENE DNA purification kit. Verification of the insertion was carried out by PCR amplification of the *AcAd* genes with the nitroAc (5'-CCACCCAACCCAATCACTACC-3') and nitroAd (5'-ATCACGAATGCCCGCCATCCA-3') primers. A larger fragment (by approximately 1 kb) was obtained from the mutant strain compared to the wild type.

Construction of the* Acidovorax *sp. JS42* cdoE *mutant. The 4.5-kb KpnI-BamHI fragment from pDTG903 (29) carrying *cdoE* from *Comamonas* sp. JS765 (which is identical in sequence to the *cdoE* gene in JS42) was inserted into KpnI-BamHI-digested pK18 (31) to generate pDTG928. The streptomycin-spectinomycin resistance cassette was excised from pHP45Ω (30) using SmaI and inserted into the unique ScaI site within *cdoE* on pDTG928, generating pDTG929. The inactivated *cdoE* gene fragment (~6.0-kb) was excised from pDTG929 using XbaI and KpnI and inserted into XbaI-KpnI-digested pRK415 (18) to form pDTG930. pDTG930 was introduced into *Acidovorax* sp. JS42 by conjugative transfer from *E. coli* S17-1 (35) as described above. A streptomycin-resistant (200 µg/ml), tetracycline-sensitive colony was obtained. The insertion of the Ω cassette in *cdoE* in the genomic DNA of this strain (JS42*cdoE*::Sm) was confirmed by Southern hybridization with the *cdoE* gene as the probe (data not shown). As expected, this strain did not grow with 2NT. Catechol 2,3-dioxygenase assays (4) were carried out with crude cell extracts of wild-type JS42 and JS42*cdoE*::Sm and catechol or 3-methylcatechol as substrates. Unlike wild type, JS42*cdoE*::Sm did not have detectable catechol 2,3-dioxygenase activity with either substrate (data not shown).

RT-PCR. *Ralstonia* sp. strains U2, U2*nagY*::Km, and U2*nagR*::Km, and *P. putida* PRS 2000(pBBR1MCS-3::*nagY*) were grown in MSB medium with 10 mM succinate (uninduced) or 10 mM succinate plus naphthalene crystals (induced), and RNA was purified from cells using the RNeasy Mini kit from Qiagen. PCR primers were designed to amplify the central 360-bp of *nagY* (based on the *nagY* sequence the primers amplify bp 437-796). The primer sequences were midnagYF, 5'-AGGCCAGCTCCCTGGAAGAAA-3' and midnagYR, 5'-TTGATTTCCTTGGCGGCCTCG-3'. RT-PCR was carried out according to the manufacturer's instructions using the OneStep RT-PCR kit from Qiagen.

Generation of cured variants of* Ralstonia *sp. U2 lacking the catabolic plasmid pWWU2. Strains U2c1 and U2c2 were generated following five sequential transfers of strain U2 in the presence of 150 µg/mL acridine orange, which was the highest concentration that allowed growth, in LB medium at 30°C overnight with shaking. The resulting culture was diluted and plated on MSB plates containing 0.5 mM succinate and naphthalene provided in the vapor phase. Small colonies were patched to MSB plates containing 10 mM succinate and MSB plates with naphthalene vapor to screen for strains unable to grow with naphthalene. Two strains (U2c1 and U2c2) that were unable to grow on naphthalene or the naphthalene degradation intermediates salicylate and gentisate were obtained. A heat-cured variant of U2*nagY*::Km (U2Hc1) was obtained after growth overnight in LB at 37^{o}C. The resulting strain did not grow with naphthalene and was kanamycin sensitive. Attempts to cure strain U2 of the naphthalene catabolic plasmid using mitomicin C, ethidium bromide, growth in the presence of fluoronaphthalene, or by introduction of potentially incompatible plasmids were not successful. None of the strains described above were able to convert indole to indigo after exposure to naphthalene, indicating that they do not possess active naphthalene dioxygenase enzymes. PCR amplification was carried out to determine whether any of the strains still carried *nagY* (1.5-kb) or *nagAcAdBF* (2.9-kb) using primers nagYF (5'-CACCACCTGGAGCGGGAACATCTT-3') and nagYR (5'-GCCAAGTACAACCGGGATCCG-3') or nagAc-FF (5'-GCTGAAGCCGGAAATGCGAAA-3') and nagAc-FR (5'-GCTTCCCGGAATACATTGGCC-3'). None of the strains carried these portions of the *nag* gene cluster, although products of the appropriate size were obtained from wild-type *Ralstonia* sp. U2. In addition, PCR amplification and sequence analysis of the 16S RNA gene from each strain was carried out to confirm that each was derived from *Ralstonia* sp. U2. The colony and cell morphologies of all of the cured strains were also consistent with those of U2.

Purification of pWWU2 plasmid DNA and construction of clone banks for sequencing. A sucrose gradient method (40) was used to purify plasmid DNA from *Ralstonia* sp. U2 cells. The purity of the plasmid DNA (ie. lack of chromosomal DNA) in each collected fraction was assessed using agarose gels and the purest fractions were pooled and used for clone bank construction. Purified plasmid DNA from strain U2 was cleaved with either BamHI or EcoRI and inserted into linearized pK18 or pK19 vectors (31). Total BamHI inserts in pK18 or pK19 clones were 239 kb and total EcoRI inserts pK18 or pK19 clones were 198 kb. With a reported size for pWWU2 of approximately 250 kb (9), we expected that this bank represented almost 2-fold coverage and would provide sufficient sequence data to cover a large percentage of pWWU2.

Plasposon mutagenesis of* Ralstonia *sp. U2. To identify the naphthalene chemoreceptor gene, we generated a bank of *Ralstonia* sp. U2 mutants with insertions in naphthalene-inducible genes by mutagenesis with a promoter probe-plasposon carrying a promoterless kanamycin resistance gene and a functional tetracycline resistance gene (a derivative of the plasposons described in (7); J.J. Dennis and G.J. Zylstra, personal communication). The plasposon pTnModpKmOTc was introduced into *Ralstonia* sp. U2, by

triparental mating from JM109 using HB101(pRK2013) to mobilize the plasposon (8). Twenty independent matings were carried out on LB medium and mating mixes were plated on MSB plates containing 10 mM succinate and 12.5 µg/ml tetracycline. Tetracycline resistant exconjugants were screened for naphthalene-inducible kanamycin resistance in order to identify those mutants with insertions in naphthalene-inducible genes. This subset of mutants was then assayed for chemotaxis to naphthalene using the swarm tube assay (see below).

Chemotaxis assays. We used qualitative and quantitative capillary assays (1, 12) to monitor chemotaxis of the two wild-type strains and relevant mutant strains after growth under different conditions. Bacterial cells were harvested in mid-exponential phase (when the optical density at 660 nm [OD_{660}] was between 0.3 and 0.5) by centrifugation at 5,000 rpm for 5 min, and washed once with chemotaxis buffer (50 mM potassium phosphate buffer [pH 7.0], 10 µM disodium EDTA, 0.05 % glycerol) (27).

For qualitative assays, microcapillaries either contained attractants in 2% low-melting-temperature agarose dissolved in chemotaxis buffer or crystals of solid attractants (e.g. naphthalene). Microcapillaries were introduced into suspensions of motile cells in chemotaxis buffer at an OD_{660} of approximately 0.1, and the response was visualized under the microscope at 40X magnification and photographed. A chemotactic response is visible as a cloud of cells that accumulates near the tip of the capillary over time.

Traditional quantitative capillary assays were carried out as previously described with slight modifications (1, 15). Capillaries (1 µl) were filled with 0.63 µl of chemotaxis buffer or an attractant dissolved in chemotaxis buffer under vacuum as previously described (23). Attractants were tested at a concentration of 250 µM (saturating for naphthalene). Washed cells were suspended in chemotaxis buffer to an OD_{660} of approximately 0.10, placed in a chamber formed by a cover slip, a glass U-tube and the bottom of a Petri dish, and a capillary was inserted into the pool of bacterial cells. After incubation for 1 h at room temperature, the capillary contents were collected, diluted, and cells were enumerated as colony forming units by plate counts on LB plates (6). In addition, we developed the traditional quantitative capillary assay into a high-throughput capillary assay, using a 96-well microtiter-plate format. One µl capillaries were sealed at one end, sterilized and inserted into the center of each well, which was pre-filled with 3% agar. Then the plate with was inverted, inserting capillaries into the wells of a new plate that contained 100 µl of chemotaxis buffer or an attractant dissolved in chemotaxis buffer. This unit was placed under vacuum to fill the capillaries as described above. The capillaries were briefly washed with MSB and then they were inserted into another plate pre-filled with 300 µl of bacterial suspension in each well, with a pipette tip tray in the middle as a spacer. After incubation of this assembly at room temperature for 1 h, the capillaries were briefly rinsed with MSB and the contents were collected, diluted, and cells were enumerated as colony forming units by plate counts on LB plates. Results are based on plate counts for each of at least 15 capillaries from at least two independent experiments. In all experiments, negative controls (chemotaxis buffer) and positive controls (aspartate or adipate) were included.

A soft agar swarm tube assay was developed for semi-high-throughput screening of potential naphthalene chemotaxis mutants with plasposon insertions. Swarm tube medium consisted of MSB containing 0.3% noble agar (Difco) and a crystal of naphthalene at the bottom of the tube. Wild-type *P. putida* G7 and the naphthalene chemotaxis mutant G7-Y1 (13) were used as positive and negative controls, respectively. Both strains grow equally well on naphthalene, but G7-Y1 is specifically nonchemotactic to naphthalene due to the deletion of the *nahY* gene (13). Cells were grown in MSB containing naphthalene overnight at 30^{o}C, diluted in MSB to an OD_{660} of 0.2, and 5 µl of cells were inoculated at the top of each tube. A band of chemotactic G7 cells formed after overnight incubation, whereas no band was observed even

following extended incubation of G7-Y1 (**Fig. 1**). Two bands of *Ralstonia* sp. U2 cells consistently formed under the same conditions (**Fig. 1**). This assay was used to screen the bank of *Ralstonia* sp. U2 plasposon mutants.

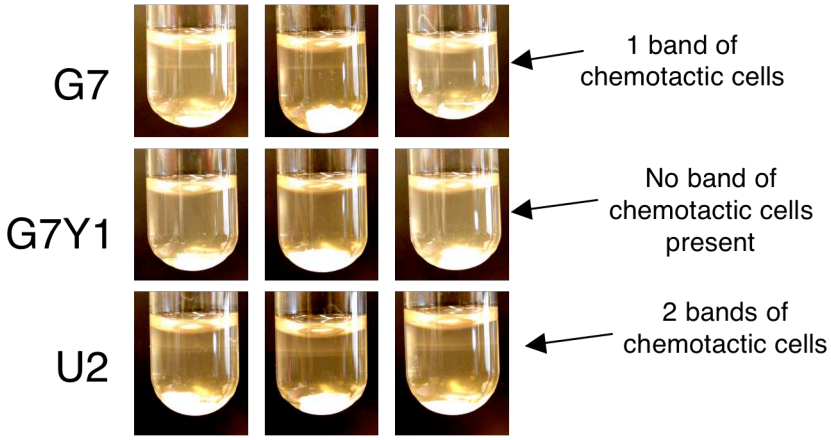

Fig. 1. Swarm tube assay. Three replicates of each strain (wild-type *P. putida* G7, *P. putida* G7-Y1 lacking the naphthalene chemoreceptor, and wild-type *Ralstonia* sp. U2) were grown overnight in MSB containing naphthalene, then diluted to O.2 OD_{660}. 5µL of each diluted culture was used to inoculate the top of the naphthalene swarm tubes.

Summary of Results

I. Chemotaxis to naphthalene by *Ralstonia* sp. strain U2

Naphthalene chemotaxis is induced during growth with naphthalene or salicylate. *Ralstonia* sp. strain U2 was grown in minimal medium with succinate (uninduced), or succinate plus naphthalene (induced), and each culture was tested in qualitative capillary assays. Naphthalene-grown U2 cells were chemotactic to naphthalene, and the positive control attractant adipate (**Fig. 2**). In contrast, succinate-grown U2 showed only responded very weakly to naphthalene, but still responded to adipate (**Fig. 2**), indicating that chemotaxis to naphthalene is inducible during growth with naphthalene.

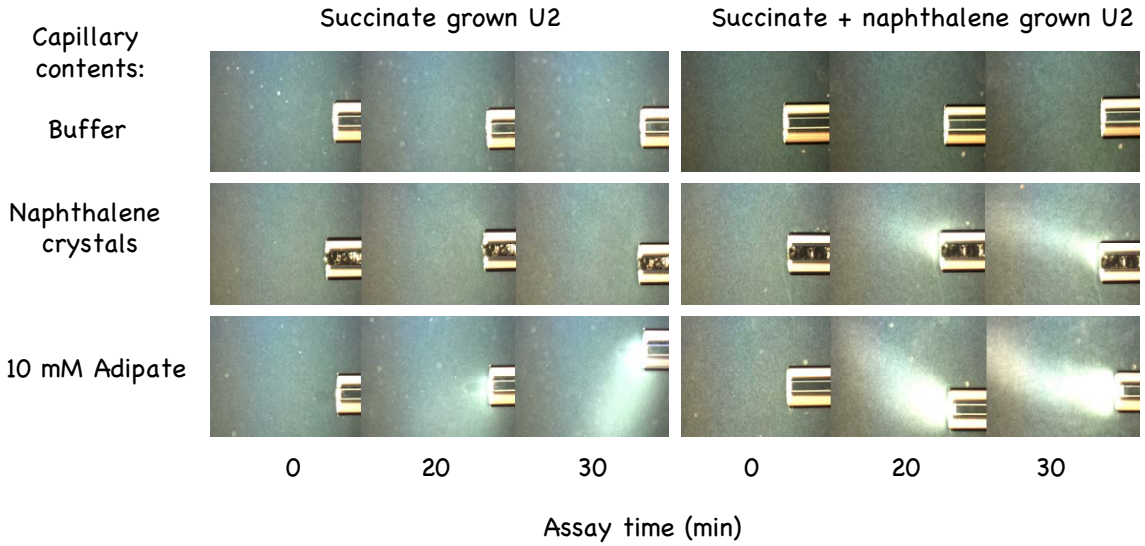

Fig. 2. Qualitative capillary assay showing chemotaxis of wild-type uninduced (succinate-grown) and induced (succinate plus naphthalene-grown) *Ralstonia* sp. U2 cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose, naphthalene crystals, or 10 mM adipate in chemotaxis buffer solidified with 2% low-melting temperature agarose.

The chemotactic response to naphthalene and the naphthalene degradation intermediates gentisate and salicylate was also tested using the quantitative capillary assay (**Fig. 3**). Clear responses to naphthalene and gentisate were detected. The response to salicylate was not significantly higher than background.

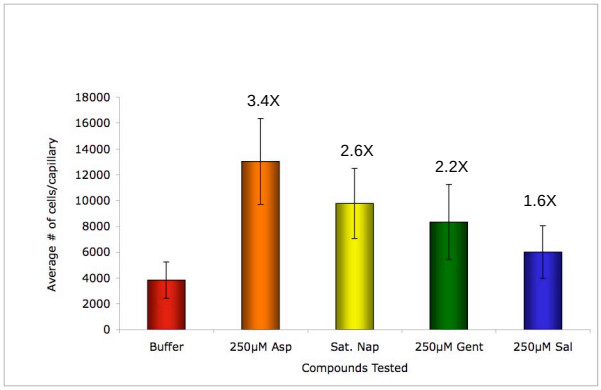

Fig. 3. Quantitative capillary assays showing chemotaxis of wild-type induced (succinate plus naphthalene-grown) *Ralstonia* sp. U2 cells. Buffer indicates the number of cells that entered a capillary containing chemotaxis buffer only (background). Attractants were used at 250 μM. Asp, aspartate (positive control); Nap, naphthalene (saturating: ~250 μM); Gent, gentisate; Sal, salicylate. Fold response over background (buffer only) is shown above each bar. Standard deviations are indicated by error bars.

Salicylate is the inducer of the naphthalene degradation genes, interacting with the positive regulator NagR to turn on the naphthalene degradation genes in strain U2 (17). Induced chemotactic responses to naphthalene were seen with salicylate-grown or succinate plus salicylate-grown wild-type U2 cells (**Table 1**). This result indicates that naphthalene degradation and chemotaxis are coordinately regulated and brought up the question of whether NagR was responsible for controlling expression of the naphthalene chemotaxis gene(s).

Table 1. Summary of chemotaxis responses of *Ralstonia* sp. U2 and mutant derivatives

		Compound Tested as Attractant		
Strain	Growth Substrate	Buffer	10 mM Adipate	Naphthalene
U2 (Wild type)	Naphthalene	-	+++	+++
	10 mM Succinate	-	+++	+
	10 mM Succinate + Naphthalene	-	+++	+++
	10 mM Succinate + 2.5 mM Salicylate	-	+++	+++
	5 mM Salicylate	-	+++	++
U2nagR::Km	10 mM Succinate	-	+++	+
	10 mM Succinate + Naphthalene	-	+++	+++
	10 mM Succinate + 2.5 mM Salicylate	-	++	+++
U2nagY::Km	10 mM Succinate	-	+++	+
	10 mM Succinate + Naphthalene	-	+++	+++
	10 mM Succinate + 2.5 mM Salicylate	-	+++	++
U2 cured	10 mM Succinate	-	++	-
	10 mM Succinate + Naphthalene	-	++	-
	10 mM Succinate + 2.5 mM Salicylate	-	++	-

Results are based on qualitative capillary assays. naphthalene was tested in capillaries as solid crystals. -, no response; +, weak response; ++, medium response; +++, strong response

NagR, the transcriptional activator of the naphthalene degradation genes is not required for induction of the naphthalene chemotactic response; naphthalene is detected directly by* Ralstonia *sp. U2. NagR is a LysR-type transcriptional activator that turns on expression of the naphthalene degradation genes in *Ralstonia* sp. U2. In order to test whether

NagR was also responsible for controlling expression of the naphthalene chemotaxis gene(s), we generated a *nagR* mutant by insertional inactivation of the wild-type *nagR* gene (see Materials and Methods). This strain, U2*nagR*::Km, was tesed for its response to naphthalene, adipate or buffer after growth with succinate (uninduced) or succinate plus naphthalene (induced). Strain U2*nagR*::Km showed wild-type chemotaxis to naphthalene (**Fig. 4**), indicating that NagR does not control expression of the naphthalene chemotaxis gene(s). The response was induced in the presence of either naphthalane or salicylate, and the strain also had a wild-type response to adipate (**Table 1**). Since the NagR mutant is unable to metabolize naphthalene, this result also demonstrates that naphthalene is directly detected as the attractant, rather than an intermediate in the naphthalene degradation pathway.

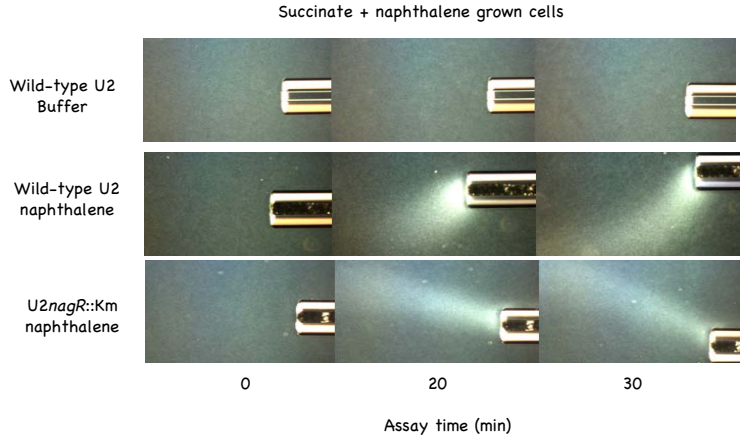

Fig. 4. Qualitative capillary assay showing chemotaxis of succinate plus naphthalene-grown *Ralstonia* sp. U2 and U2*nagR*::Km cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose, or naphthalene crystals. Both strain responded to adipate controls (data not shown).

The naphthalene chemoreceptor is encoded on a plasmid in* Ralstonia *sp. U2. The genes encoding the naphthalene degradation pathway are located on the large catabolic plasmid pWWU2. In our hands, the plasmid is very stable in strain U2 and is not lost during extended growth on alternative carbon sources, rich medium, or in the presence of the curing agents mitomycin C, ethidium bromide, or with 1-fluoronaphthalene, which was shown to induce the loss of the naphthalene catabolic plasmid pDTG1 from *Pseudomonas* sp. NCIB9816-4 (34). We were able to generate cured strains lacking pWWU2 by growing U2 in the presence of acridine orange or by growth at higher temperatures (see Materials and Methods). The cured strains U2c1, U2c2, and U2Hc1 did not grow on naphthalene, salicylate, or gentisate and pWWU2 could not be isolated from the strains using a method that allowed isolation of the plasmid from the wild-type strain. The absence of the plasmid was further verified by PCR amplification to detect genes known to be on pWWU2 (*nagY* and *nagAc-F*; data not shown). The cured strains did not respond to naphthalene in qualitative assays (**Fig. 5; Table 1**; data not shown), indicating that the naphthalene chemoreceptor is likely to be encoded on the plasmid.

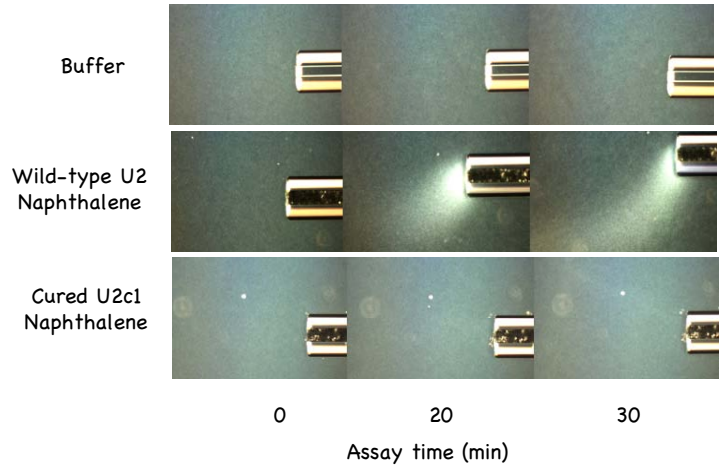

Fig. 5. Qualitative capillary assay showing chemotaxis of succinate plus naphthalene-grown wild-type *Ralstonia* sp. U2 and cured *Ralstonia* sp. U2c1 cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose, or naphthalene crystals. Both strain responded to adipate controls (data not shown).

New protein synthesis is required for chemotaxis to naphthalene. In order to confirm that new protein synthesis was required for the inducible chemotaxis response, *Ralstonia* sp. U2 cells were induced in the presence of naphthalene and chloramphenicol for 2.5 h prior to harvesting and qualitative assays. Preliminary experiments indicated that 2.5 hours was the minimum time required for induction of the naphthalene chemotactic response. These cells did not respond to naphthalene but did respond to the control attractant adipate (**Fig. 6**). Control cells exposed to naphthalene in the absence of chloramphenicol for the same amount of time did respond to naphthalene (not shown).

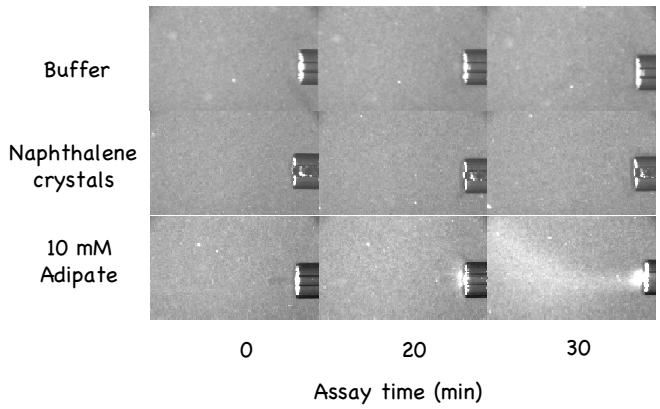

Fig. 6. Chemotactic response of *Ralstonia* sp. U2 following induction with naphthalene in the presence of chloramphenicol. Cells were grown with succinate. Naphthalene and 20 µg/ml chloramphenicol were added 2.5 h prior to harvesting cells for assays. Control cells exposed to naphthalene for 2.5 h in the absence of chloramphenicol responded to both naphthalene and adipate (not shown).

NagY is not a naphthalene chemoreceptor. In earlier work, we identified a putative chemoreceptor gene (*nagY*) upstream of the naphthalene catabolic genes on pWWU2 and proposed that it might encode the naphthalene chemoreceptor. The *nagY* gene is located in the gene cluster for naphthalene degradation in *Ralstonia* sp. U2 (41), and based on its sequence, appears to encode a methyl-accepting chemotaxis protein. The partial *nagY* gene had high sequence identity (96%) to the *nbzY* gene from *Comamonas* sp. JS765 (20). The complete *nagY* gene had not been cloned and sequenced, so we designed primers based on *nbzY* and amplified the complete *nagY* gene by PCR. In order to determine the role of *nagY* in chemotaxis to naphthalene, we generated a *nagY* mutant by insertional inactivation of *nagY* with a kanamycin resistance gene. The insertion in this strain (U2*nagY*::Km) was verified by PCR. Strain U2*nagY*::Km grew on naphthalene like wild-type U2. Unexpectedly, it responded to naphthalene in qualitative assays (**Fig. 7 and Table 1**). These results indicate that either 1) NagY is not the chemoreceptor for naphthalene or 2) an additional chemoreceptor with overlapping specificity is present in strain U2. Using qualitative capillary assays we also showed that the U2*nagY*::Km mutant has a wild-type response to gentisate (data not shown).

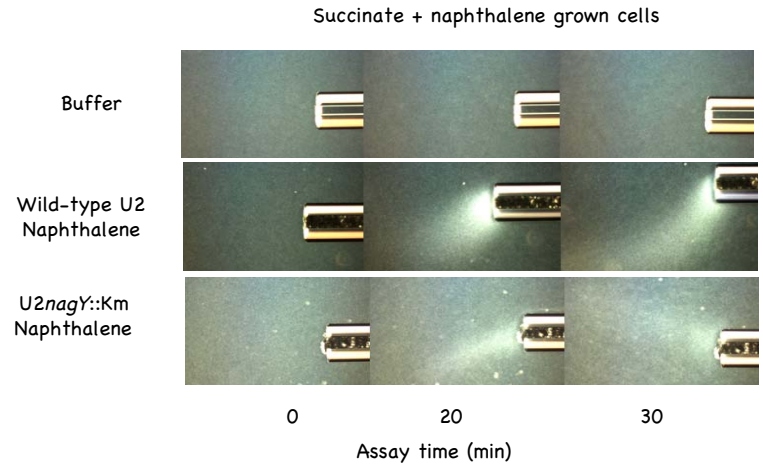

Fig. 7. Chemotactic response of succinate plus naphthalene grown *Ralstonia* sp. U2 and U2*nagY*::Km. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose, or naphthalene crystals. Both strain responded to adipate controls.

We used RT-PCR to verify that *nagY* is expressed in *Ralstonia* sp. U2 (**Fig. 8** lanes 3-5) and that a functional transcript is not made in the *nagY* mutant (**Fig. 8** lanes 6-8). These studies also demonstrated that *nagY* was expressed equally well in cells grown in the presence or absence of naphthalene (**Fig. 8** lanes 3-5), indicating that *nagY* cannot be directly responsible for inducible naphthalene taxis in U2. In addition, *nagY* was expressed in *nagR* mutant cells (**Fig. 8**, lanes 9 and 10), confirming that *nagY* expression is not under the control of NagR.

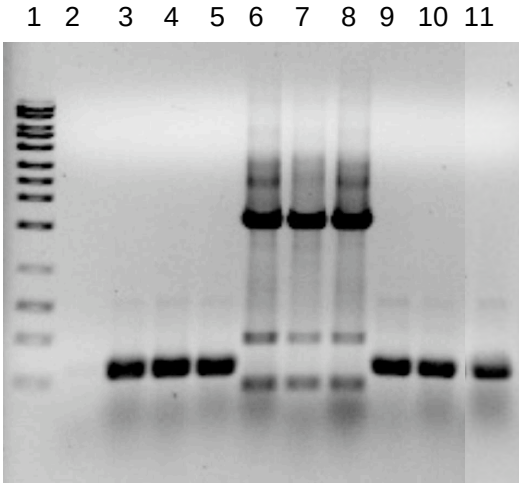

Fig. 8. Agarose gel of RT-PCR products. Primers were designed to amplify the central part of *nagY* (~360 bp). Lanes: 1, 1 kb ladder (the smallest band is 300 bp); 2, no template RNA control; 3, U2 RNA from naphthalene-grown cells; 4, U2 RNA from succinate-grown cells; 5, U2 RNA from succinate + naphthalene-grown cells; 6, U2 *nagY*::Km RNA from naphthalene-grown cells; 7, U2 *nagY*::Km RNA from succinate-grown cells; 8, U2 *nagY*::Km RNA from succinate + naphthalene-grown cells; 9, U2 *nagR*::Km RNA from succinate + naphthalene-grown cells; 10, U2 *nagR*::Km RNA from succinate-grown cells; 11, PRS2000(pBBR1MCS-3::*nagY*) RNA from succinate + naphthalene-grown cells. No products were obtained when reverse transcriptase was omitted from the reaction mixtures (not shown).

Above we showed that chemotaxis to naphthalene required the presence of the catabolic plasmid because a cured strain lacking pWWU2 (U2c1) did not respond to naphthalene (**Fig. 5**). We cloned the *nagY* gene onto a broad-host-range plasmid and introduced it into the cured variant of U2 to test whether NagY conferred the ability to respond to naphthalene. Introduction of the cloned *nagY* gene into the cured strain did not complement the chemotaxis defect (data not shown). We also tested whether NagY could confer the ability to respond to naphthalene in alternative strain backgrounds by introducing the gene into *Pseudomonas putida* PRS2000, a strain that is known to be chemotactic to aromatic acids but not to naphthalene, and a mutant of *P. putida* G7 that has had its naphthalene chemoreceptor gene *nahY* inactivated (G7-Y1). *nagY* expression was demonstrated in *P. putida* PRS2000 using RT-PCR (**Fig. 8**, lane 11), but the presence of NagY did not confer the ability to respond to naphthalene in this strain or in the G7 *nahY* mutant (not shown). From these results, we concluded that *nagY* does not encode a chemoreceptor for naphthalene.

The naphthalene chemoreceptor in* Ralstonia *sp. U2 is different from that in P. putida G7. The chemoreceptor for naphthalene chemotaxis in *P. putida* G7 (NahY) has been identified (13). In order to determine whether *Ralstonia* sp. U2 carried a similar receptor, we obtained the *nahY* gene and carried out Southern blots with *Ralstonia* sp. U2 genomic DNA. No hybridizing bands were observed under any of the tested conditions (not shown), suggesting that the naphthalene chemoreceptors are not alike in the two strains.

The continuing search for the naphthalene chemoreceptor. As mentioned above, the genes encoding the naphthalene degradation pathway are located on the large catabolic plasmid pWWU2. An early report in the literature had indicated that this plasmid was approximately 250-kb in size and no mention was made of the presence of other plasmids in strain U2 (9). We decided to sequence the plasmid in order to facilitate the identification of the naphthalene chemoreceptor gene. Plasmid DNA was purified by using a sucrose gradient (40), and we generated two clone banks containing a total of 435 kb of plasmid DNA inserts. The clone banks were provided to Dr. Gerben Zylstra at Rutgers University for sequencing. >200 kb

of sequence data was obtained, and only one sequence matched the 25-kb *nag* catabolic gene region known to be present on pWWU2. Based on the sequence data, it appears that multiple large plasmids are present in U2 and the *nag* genes are carried on a large low-abundance plasmid; most of the sequence appeared to be from another plasmid and its sequence was approximately 95% completed. The sequence of pWWU2 could not completed from these clone banks. Regardless, we analyzed all of the available sequence data but did not identify any likely candidates for a naphthalene chemoreceptor gene.

As an alternative method to identify the naphthalene chemoreceptor, we carried out transposon mutagenesis of *Ralstonia* sp. U2 using a plasposon carrying a tetracycline resistance gene and a promoterless kanamycin resistance gene (7). So far, we generated and screened >16,000 plasposon mutants for strains that demonstrated naphthalene-inducible kanamycin resistance, which would indicate that the plasposon had inserted in a naphthalene-inducible gene or operon. We identified 18 strains that were not capable of growth on naphthalene but showed naphthalene-inducible kanamycin resistance. The insertions in these strains were expected to be in the *nag* catabolic operon. We identified 2335 mutants that were capable of growth on naphthalene and also showed naphthalene-inducible kanamycin resistance. This class of mutants is expected to contain mutants with insertions in the naphthalene chemoreceptor gene. Qualitative capillary assays were initially used to screen for loss of chemotaxis to naphthalene following growth on naphthalene. In order to speed the screening process, we developed the swarm tube assay (see Materials and Methods) and screened the majority of the mutants with this assay. However, none of the mutants with insertions in naphthalene-inducible genes lost the ability to respond to naphthalene. We have used arbitrary PCR to amplify the plasposon and flanking DNA in order to identify the position of the insertions in the 18 strains that were no longer able to grow on naphthalene, and none of the insertions were in the identified 25-kb catabolic gene cluster for naphthalene degradation, suggesting that we have not yet saturated the *Ralstonia* sp. U2 genome with plasposon insertions. Once we do identify an insertion that eliminates chemotaxis to naphthalene, the wild-type region will be cloned, sequenced, and used to complement the mutant.

II. Chemotaxis of *Acidovorax* sp. JS42

Acidovorax *sp. JS42 has two types of responses to 2NT: a low constitutive response and a stronger 2NT-inducible response*. *Acidovorax* sp. JS42 was grown in minimal medium with succinate (uninduced) or with succinate plus 2NT (induced), and chemotaxis was tested with qualitative capillary assays. Qualitative capillary assay results demonstrated a strong response to 2NT by induced cells, and a weaker response by uninduced cells (**Fig. 9**). These results suggest that JS42 has a low constitutive response to 2NT, but induces some essential chemotaxis gene(s) when grown in the presence of 2NT.

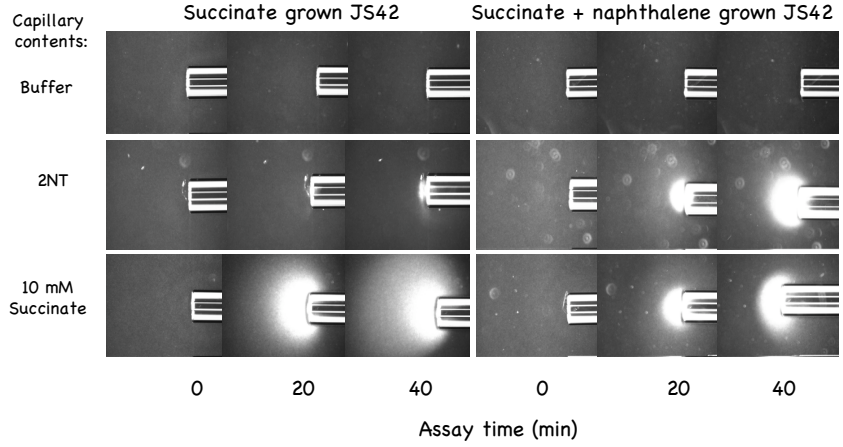

Fig. 9. Qualitative capillary assays showing chemotaxis of wild-type uninduced (succinate-grown) and induced (succinate plus 2NT grown) *Acidovorax* sp. JS42 cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose alone, or containing 4.4 mM 2NT (saturated), or 10 mM succinate.

The inducible chemotactic response to 2NT requires the ability to metabolize 2NT in* Acidovorax *sp. JS42. In order to determine whether 2NT metabolism is required for the response to 2NT, we generated two mutant strains that are unable to degrade 2NT (JS42*ntdAc*::Km and JS42*cdoE*::Sm). JS42*ntdAc*::Km cannot grow on 2NT because it is blocked at the initial step of 2NT catabolism; it has an inactivated 2NT dioxygenase alpha subunit gene. However, JS42*ntdAc*::Km remains capable of growth on the product of 2NT oxidation, 3-methylcatechol. JS42*cdoE*::Sm has an inactivated 3-methylcatechol dioxygenase, and is therefore blocked in the second step (aromatic ring cleavage) in 2NT degradation; this strain cannot grow on 2NT or 3-methylcatechol. JS42*ntdAc*::Km cells remained capable of the low constitutive response to 2NT but did not mount the strong induced response (**Fig. 10**). These results suggest that 2NT is directly detected by a constitutively expressed chemoreceptor but metabolism is required for the inducible response. Similarly, the response of JS42*cdoE*::Sm to 2NT was not inducible (**Table 2**).

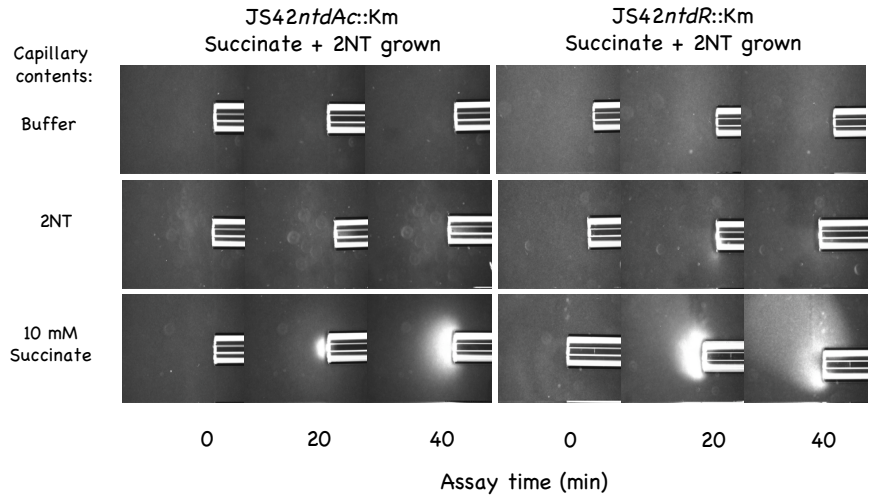

Fig. 10. Qualitative capillary assays showing chemotaxis of induced (succinate plus 2NT-grown) *Acidovorax* sp. JS42*ntdAc*::Km and JS42*ntdR*::Km cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose alone, or containing 4.4 mM 2NT (saturated), or 10 mM succinate.

Inactivation of the transcriptional activator NtdR in* Acidovorax *sp. JS42 eliminated the inducible chemotactic response to 2NT. *ntdR* encodes a LysR-type transcriptional activator that is required for expression of the genes encoding 2NT dioxygenase (the first enzyme in the 2NT degradation pathway) and for growth on 2NT (20). JS42*ntdR*::Km is a regulatory mutant that is unable to induce the 2NT degradation genes (20). JS42*ntdR*::Km cells retained the low constitutive response to 2NT but were uninducible for strong 2NT chemotaxis (**Fig. 10**). These results provide additional evidence that 2NT is directly detected but that metabolism is required for the stronger inducible response. This inducible response may actually be an example of energy taxis, where the cells are responding to chemicals that increase the energy state of the cell (such as good carbon sources or alternative electron acceptors) rather than to a specific chemical attractant (3, 39).

Another explanation for the inducible chemotactic response could be that the cells are responding to a 2NT metabolite rather than 2NT itself, and NtdR could be required for the expression of the chemoreceptor gene in response to either 2NT or a 2NT metabolite. Therefore, we tested the products of 2NT metabolism, 3-methylcatechol, 2-nitrobenzyl alcohol, and nitrite, as chemoattractants. 3-Methylcatechol served as an attractant for wild-type JS42, JS42*ntdAc*::Km, and JS42*ntdR*::Km and the response was not dependent on the growth substrate provided (**Table 2**). In contrast, JS42*cdoE*::Sm, which cannot grow on 3-methylcatechol, was not attracted (**Table 2**). Similar results were obtained with 4-

methylcatechol, which is also a growth substrate for JS42, JS42*ntdAc*::Km, and JS42*ntdR*::Km, but not JS42*cdoE*::Sm (data not shown). These results are consistent with the possibility that the responses to 3- and 4-methylcatechol may be the result of energy taxis. Nitrite, which is formed along with 3-methylcatechol during the oxidation of 2NT, was found to serve as an attractant for JS42 and all of its mutant derivatives, regardless of prior growth conditions (**Table 2**), suggesting that the response to nitrite is unrelated to the responses to 2NT. In addition, we tested 2-nitrobenzyl alcohol (4.4 mM or crystals) as an attractant since JS42 forms small amounts of this compound from 2NT as a dead-end metabolite (25). 2NT-induced JS42 did not respond to 2-nitrobenzylalcohol (data not shown).

Table 2. Summary of *Acidovorax* chemotactic responses

		Compound in capillary						
Strain	Growth Substrate(s)	Buffer	Succinate	2NT	3NT	4NT	3MC	Nitrite
JS42 (Wild type)	Succinate	-	+++	+	+	-	+	+
	Succinate + 2NT	-	+++	+++	+	-	+	+
	Succinate + 3MC	-	+++	++	+	-	+	+
JS42*ntdAc::Km*	Succinate	-	++	+	+	-	++	+
	Succinate + 2NT	-	+++	+	+	-	+	+
	Succinate + 3MC	-	+++	+	+	-	+	+
JS42*ntdR::Km*	Succinate	-	+++	+	+	-	+	+
	Succinate + 2NT	-	++	+	+	-	+	++
	Succinate + 3MC	-	+++	+	+	-	+	++
JS42*cdoE::Sm*	Succinate	-	+++	++	+	-	-	+
	Succinate + 2NT	-	++	++	+	-	-	+
	Succinate + 3MC	-	+++	++	+	-	-	+

Results are based on qualitative capillary assays. For growth substrates, succinate was provided at 10 mM and 2NT and 3-methylcatechol (3MC) were provided at 1.5 mM. As attractants, succinate and 3MC were provided in the capillary at 10 mM, 2NT and 3NT were provided at saturating concentrations (~4 mM), nitrite was provided at 0.1 mM, and 4NT was tested in capillaries as solid crystals. -, no response; +, weak response; ++, medium response; +++, strong response

The chemotactic responses of JS42 seem to be much more complicated than previously thought. Based on our current results, it appears that there is a specific constitutively expressed chemoreceptor for 2NT, which also detects 3NT. The inducible response to 2NT in strains capable of growth on 2NT, as well as the constitutive responses to 3- and 4-methylcatechol may be due to energy taxis. Finally, JS42 and its derivatives also respond to nitrite (a useful nitrogen source for this organism) by a third and unrelated mechanism.

Evidence that the inducible 2NT response is a form of energy taxis. All of our work to date indicates that the inducible response to 2NT and the 2NT metabolite 3-methylcatechol (3MC) requires metabolism. We have proposed that this is an energy taxis response. Energy taxis is the ability of bacteria to respond to changes in their energy generating processes in order to seek environments that support maximum energy production (39). This behavior differs from the classical chemotactic response in which the binding of a specific chemical to the periplasmic domain of a chemoreceptor is the signal for a behavioral response. The *E. coli* Aer protein is the most well studied energy taxis transducer. Unlike other chemoreceptors, Aer contains a conserved PAS domain that binds FAD (38). In addition to guiding cells in oxygen gradients, Aer was found to mediate motility in gradients of rapidly oxidizable substrates by

detecting the redox status of bound FAD, which can accept electrons from the electron transport system.

Specific chemoreceptor proteins that detect chemicals directly often detect closely related structural analogues whether they are metabolized or not. However, nonmetabolizable analogues would not be detected by an energy taxis system. We tested the two nonmetabolizable mononitrotoluene isomers, 3-nitrotoluene (3NT) and 4-nitrotoluene (4NT) as attractants for *Acidovorax* sp. JS42 and its mutant derivatives. Neither of these 2NT structural analogues serves as a carbon source for wild-type JS42. Induced and uninduced wild-type and mutant JS42 strains responded weakly to 3NT, but not to 4NT (**Table 2**). Neither compound elicited the strong inducible response (**Table 2**), which is consistent with the idea that the response 2NT to is due to energy taxis.

The gratuitous inducer of the 2NT dioxygenases, salicylate, induces the strong chemotactic response to 2NT in wild-type JS42 but not in the metabolic or regulatory mutants JS42*ntdAc*::Km and JS42*ntdR*::Km (**Fig. 11**). This data provides further evidence that the inducible response to 2NT is directly connected to active metabolism of 2NT, providing additional support for an energy taxis response.

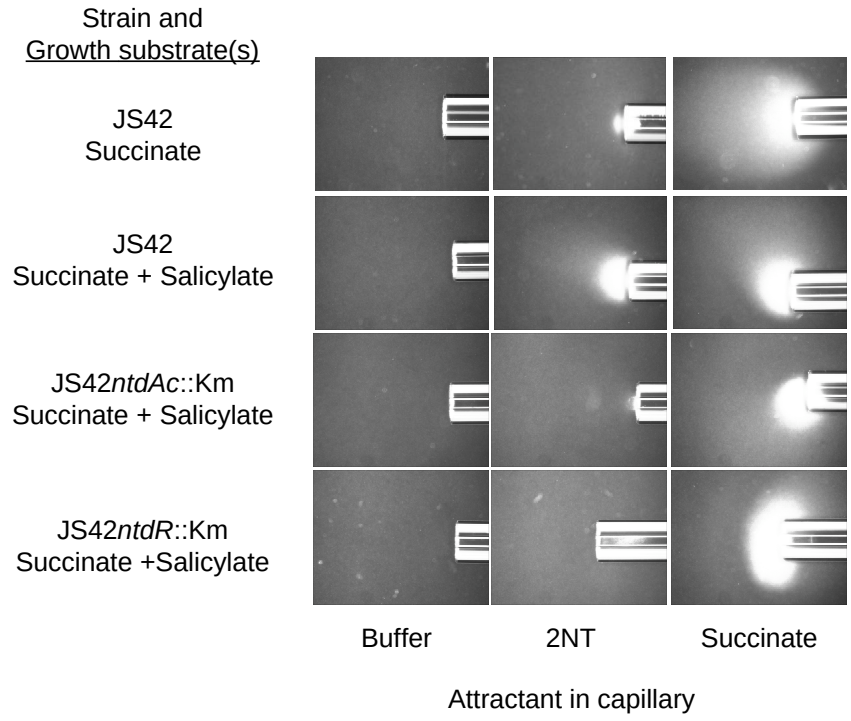

Fig. 11. Qualitative capillary assays showing chemotaxis of succinate-grown (uninduced) and induced (succinate plus salicylate-grown) *Acidovorax* sp. JS42, JS42*ntdAc*::Km and JS42*ntdR*::Km cells. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose alone, or containing 4.4 mM 2NT (saturated), or 10 mM succinate. Photographs were taken at the peak of the response (20-40 min).

To gather additional support for the role of energy taxis, we carried out competition assays (2). In these assays, cells were suspended in buffer containing the competing attractant, succinate. The test attractant (e.g. 2NT) was provided in the capillary. If the cells detect 2NT directly via a specific receptor, they should still respond. However, if the cells are carrying out energy taxis, there will be no induced response because they are already metabolizing succinate and should be in a fully energized state. Results of these assays support the proposal that JS42 senses 2NT via energy taxis, as responses to 2NT were reduced or completely eliminated when succinate was present in the chamber with the cells (**Fig. 12; Table 3**).

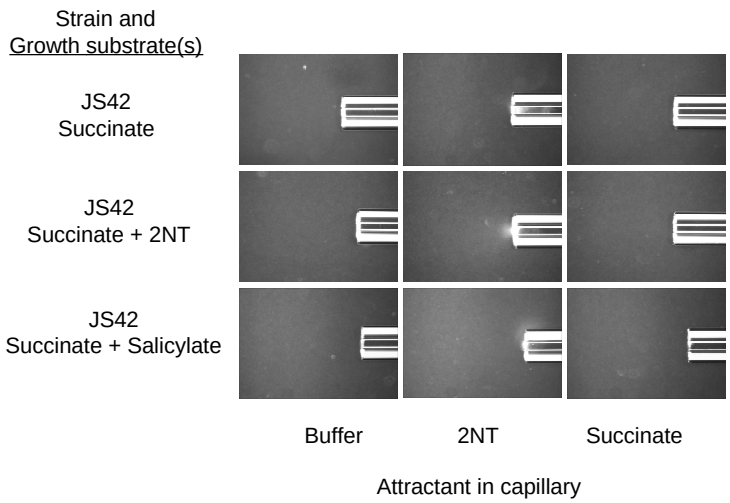

Fig. 12. Qualitative competition assays showing chemotaxis of uninduced (succinate grown) and induced (succinate plus 2NT or succinate plus salicylate grown) wild-type *Acidovorax* sp. JS42 cells. In these experiments the competing attractant succinate (10 mM) was also present in the cell suspension. Capillaries contained chemotaxis buffer solidified with 2% low-melting temperature agarose alone, or containing 4.4 mM 2NT (saturated), or 10 mM succinate. Photographs were taken at the peak of the response (20-40 min).

Table 3. Summary of qualitative competition assay results

		Attractant in capillary				
Strain	Growth Substrate	Buffer	Succinate	2NT	Succinate + 2NT	Nitrite
JS42 (Wild type)	Succinate	-	-	+	+	+
	Succinate + 2NT	-	-	+	+	+
	Succinate + 3MC	-	-	+	+	+
JS42*ntdAc*::Km	Succinate	-	-	+	+	+
	Succinate + 2NT	-	-	+	+	+
	Succinate + 3MC	-	-	+	+	+
JS42*ntdR*::Km	Succinate	-	-	+	+	+
	Succinate + 2NT	-	-	+	+	++
	Succinate + 3MC	-	-	+	+	++
JS42*cdoE*::Sm	Succinate	-	-	+	+	+
	Succinate + 2NT	-	-	+	+	+
	Succinate + 3MC	-	-	+	+	+

Results are based on qualitative competition capillary assays. For growth substrates, succinate was provided at 10 mM and 2NT and 3-methylcatechol (3MC) were provided at 1.5 mM. As attractants, succinate and 3MC were provided in the capillary at 10 mM, 2NT was provided at saturating concentration (~4 mM), and nitrite was provided at 0.1 mM. In each assay the cell suspension contained 10 mM succinate as a competing attractant. -, no response; +, weak response; ++, medium response.

2NT chemotaxis and energy taxis receptors. The genome of JS42 has now been completely sequenced, and we have carried out a bioinformatic analysis of the chemotaxis machinery and the chemoreceptors present. The strain has a conserved set of cytoplasmic chemotaxis genes that are likely to function like those of the well-studied *E. coli* chemotaxis system. The genome also contains 19 genes that are predicted to encode methyl accepting chemotaxis proteins (MCPs), compared to 5 in *E. coli*. In *E. coli*, MCPs are membrane bound and function as primary receptors and signal transducers to mediate chemotaxis or energy taxis. We searched for those with structural and sequence homology with Aer, the aerotaxis/energy taxis receptor that has been studied in *E. coli* and *P. putida*. Aer has a PAS domain in the N-terminus, a conserved signaling domain in its C-terminus and a membrane

anchor between the two domains (37). Two candidate genes for an energy taxis receptor were identified, AJS0049 and AJS3295. AJS3295 has a similar structure to Aer, with one PAS domain, and two transmembrane domains that are too close to each other to allow a periplasmic loop and most likely function as an anchor, and a C-terminal signaling domain. The deduced amino acid sequence is approximately 39% identical to *E. coli* Aer. AJS0049 has two PAS domains and a C-terminal MCP signaling domain; there is no apparent transmembrane domain or membrane anchor so it appears to be located in the cytoplasm. The deduced amino acid sequence is approximately 30% identical to *E. coli* Aer.

The genome also has several candidates for a specific 2NT chemoreceptor. We decided to initially focus on two genes, AJS3087 and AJS3664. We had previously identified and targeted AJS3087 (*ntdY*) as good candidate to encode the 2NT chemoreceptor because is located immediately upstream of the 2NT degradation genes in JS42. *ntdY* encodes a protein with one transmembrane domain and a conserved C-terminal signaling domain. It is expected to have an N-terminal periplasmic domain, but the structure differs from typical *E. coli* chemoreceptors because of the absence of a second transmembrane domain. AJS3664 has a typical structure for an *E. coli* MCP and it has some homology (25% overall sequence identity) with an MCP from *P. fluorescens* that detects nitrobenzoate (*nbaY*) (16).

We cloned and sequenced the complete *ntdY* gene from *Acidovorax* sp. JS42. *ntdY* was insertionally inactivated with a kanamycin cassette, and this construct was successfully used to generate a mutation in the *nagY* homologue in *Ralstonia* sp. U2 (see above). Despite significant effort, we have been unable to generate a *ntdY* mutation in *Acidovorax* sp. JS42 using this construct and are now using a *sacB* counterselection system to generate the mutation (22). We have been successful using this method in several *Pseudomonas* strains in the lab. If successful in JS42, this method will be used to make all future gene knock-outs in this strain. We have initially targeted these four genes for inactivation and will test mutant strains for the ability to sense and respond to 2NT, 3MC, succinate and other chemoattractants. We expect that a mutant with a defect in the specific 2NT chemoreceptor will still show the 2NT-inducible response to 2NT but no response when grown with succinate. We expect that a mutant in the receptor for energy taxis will show only the low uninduced response to 2NT, but no inducible response. If we are able to identify both the specific 2NT receptor and the receptor mediating metabolism-dependent taxis, we will then construct a double mutant, which we expect would not respond to 2NT under any conditions.

Conclusions

In this study, we characterized the chemotactic responses of the two environmentally relevant biodegradative strains *Ralstonia* sp. U2 and *Acidovorax* sp. JS42. We determined the growth conditions that control the response, and the range of related chemicals that are detected. We demonstrated that the toxic pollutants naphthalene and 2NT are directly detected by these bacterial strains. All of our previous work had suggested that the 2NT-gene cluster in *Acidovorax* sp. JS42 evolved from a naphthalene degradation gene cluster very similar to that in *Ralstonia* sp. U2. This evidence includes 1) high sequence homology (~95% identity), 2) similar gene organization, 3) the presence of nonfunctional pseudogenes in the *ntd* cluster that appear to have originated from a naphthalene gene cluster like that in strain U2, 4) overlapping substrate specificity of the initial dioxygenases although the JS42 dioxygenase acquired the novel ability to oxidize 2NT with release of nitrite, 5) similar LysR regulators (98% identity) controlling dioxygenase gene expression, and 6) overlapping inducer recognition profiles of the regulatory proteins, although that of NtdR from JS42 had broadened to include nitroarene inducers (20, 24-26). We had initially hypothesized that NagY was the naphthalene chemoreceptor in *Ralstonia* sp. U2, but found that this was not the case. We had predicted that

NagY would detect naphthalene in strain U2 and that the closely homologous NtdY, which differs from NagY by 18 only amino acids, might have a broadened chemoattractant recognition profile and detect naphthalene and nitroarene attractants. However, we have not yet been able to test this due the inability to generate a *ntdY* mutant. We have shown, however, that the chemotactic response to 2NT in *Acidovorax* sp. strain JS42 is much more complicated than expected, and that two types of responses occur, a constitutive, metabolism-independent response and an inducible, metabolism-dependent response that we believe is a form of energy taxis. There is now precedent for energy taxis to aromatic compounds, as the environmental pollutant phenol was recently reported to elicit an energy taxis response in *Pseudomonas* (33). Both chemotaxis and energy taxis responses may be important for efficient biodegradation of environmental pollutants at contaminated sites, as both types of mechanisms bring biodegradative bacteria closer to the source of the pollutants they are degrading.

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